

## Induction of Apoptosis by X-linked Ectodermal Dysplasia Receptor via a Caspase 8-dependent Mechanism\*

Received for publication, July 1, 2004, and in revised form, July 22, 2004  
Published, JBC Papers in Press, July 26, 2004, DOI 10.1074/jbc.M407363200

Suwan K. Sinha and Preet M. Chaudhary†

From the Hamon Center for Therapeutic Oncology Research and the Division of Hematology-Oncology,  
University of Texas Southwestern Medical Center, Dallas, Texas 75390-8593

X-linked ectodermal dysplasia receptor (XEDAR) is a recently isolated member of the tumor necrosis factor receptor family that is highly expressed during embryonic development and binds to ectodysplasin-A2 (EDA-A2). In this report, we demonstrate that although XEDAR lacks a death domain, it nevertheless induces apoptosis in an EDA-A2-dependent fashion. The apoptosis-inducing ability of XEDAR is dependent on the activation of caspase 8 and can be blocked by its genetic and pharmacological inhibitors. Although XEDAR-induced apoptosis can be blocked by dominant-negative Fas-associated death domain (FADD) protein and FADD small interfering RNA, XEDAR does not directly bind to FADD, tumor necrosis factor receptor-associated death domain (TRADD) protein, or RIP1. Instead, XEDAR signaling leads to the formation of a secondary complex containing FADD, caspase 8, and caspase 10, which results in caspase activation. Thus, XEDAR belongs to a novel class of death receptors that lack a discernible death domain but are capable of activating apoptosis in a caspase 8- and FADD-dependent fashion. XEDAR may represent an early stage in the evolution of death receptors prior to the emergence of the death domain and may play a role in the induction of apoptosis during embryonic development and adult life.

The death receptors of the tumor necrosis factor receptor (TNFR)<sup>1</sup> superfamily and their ligands have been recognized to play a crucial role in the normal development and regulation of immune and inflammatory response (1, 2). The apoptosis-inducing

ability of these receptors has been mapped to a conserved cytoplasmic domain of 60–80 amino acids called the death domain (DD) (1, 3). TNFR1 is the prototypical and perhaps the best characterized death receptor (4). Recent studies suggest that ligand-induced trimerization of TNFR1 leads to the recruitment of DD-containing adaptor protein TRADD to a plasma membrane-bound complex (complex I) (5–7). TRADD helps in the recruitment of DD-containing serine/threonine kinase RIP1 and adaptor protein TRAF2 (5–7). Assembly of complex I occurs in lipid rafts and leads to NF- $\kappa$ B activation via RIP1-mediated recruitment of the I $\kappa$ B kinase complex, whereas JNK is activated via TRAF2-mediated activation of MAP3 kinase (5, 6). Subsequently, TRADD and RIP1 dissociate from complex I and associate with a cytoplasmic complex (complex II) consisting of DD-containing protein FADD and procaspase 8, the apical caspase of the caspase cascade (5). Under conditions favoring TNFR1-induced apoptosis, procaspase 8 is activated upon recruitment to complex II and subsequently results in the activation of downstream caspases, such as caspase 3, 6, and 7, and eventual cell death (5). Unlike TNFR1, signaling via Fas, DR4, and DR5 delivers a strong and rapid proapoptotic signal (3, 8, 9). Ligand binding to these receptors leads to DD-mediated recruitment of FADD directly without the involvement of TRADD (8–10). FADD subsequently leads to the recruitment and activation of procaspase 8 (3, 8).

Ectodysplasin A is a distantly related ligand of the TNF family that plays a key role in ectodermal differentiation (11). Mutations in the ectodysplasin gene (*Eda*) cause X-linked hypohidrotic ectodermal dysplasia, which is characterized by the absence or deficient function of hair, teeth, and sweat glands (12, 13). Several alternatively spliced transcripts of EDA have been identified (14–16). The two predominant splice variants, EDA-A1 and EDA-A2, differ from each other by a 2-amino acid motif and bind to distinct receptors (17). Thus, EDA-A1 binds to a TNF family receptor designated EDAR, whereas EDA-A2 binds to the related receptor, XEDAR (17). Transgenic expression of a secreted form of EDA-A2 resulted in thin and listless animals, which died within 1 month of birth (18). Histological examination of EDA-A2-transgenic animals exhibited multifocal myodegeneration (18). The downstream events culminating in EDA-A2-induced myodegeneration and early lethality are unclear at present.

Unlike most TNFR family receptors, XEDAR is a type III transmembrane protein (lacking an NH<sub>2</sub>-terminal signal peptide) that bears 32% sequence homology with EDAR in the extracellular ligand-binding domain (17). However, XEDAR possesses a unique intracellular region with no significant homology to other TNFRs. Two predominant alternatively spliced isoforms of XEDAR have been described, XEDAR-s and XEDAR-L, which differ from each other by the presence of a 21-amino acid linker in the juxtamembrane region of the cytoplasmic domain (19). Both XEDAR isoforms lack a death do-

\* This work was supported by Grant 1R01 DE 15189-01 from the National Institutes of Health and Grant DAMD17-02-1-590 from the Department of Defense Breast Cancer Research Program, which is managed by the United States Army Medical Research and Materiel Command. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Hillman Cancer Center, 5117 Centre Ave., Suite 1.19A, Pittsburgh, PA 15213-1863. Tel.: 412-623-7703; Fax: 412-623-1415; E-mail: chaudharypm@upmc.edu.

<sup>1</sup> The abbreviations used are: TNFR, tumor necrosis factor receptor; TNF, tumor necrosis factor; DD, death domain; DR, death receptor; TRADD, tumor necrosis factor receptor-associated death domain; JNK, c-Jun NH<sub>2</sub>-terminal kinase; FADD, Fas-associated death domain; DN-FADD, dominant-negative Fas-associated death domain; EDA, ectodysplasin A; EDAR, ectodermal dysplasia receptor; XEDAR, X-linked ectodermal dysplasia receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; RIP, receptor-interacting protein; cFLIP, cellular FLICE-inhibitory protein; FLICE, Fas-associated death domain-like interleukin-1-converting enzyme; AFC, 7-amino-4-trifluoromethyl coumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Boc, *t*-butoxycarbonyl; fmk, fluoromethyl ketone; Z-, benzyloxycarbonyl; siRNA, small interfering RNA.

main and have been shown to signal mainly via TRAF6 and TRAF3 to activate the NF- $\kappa$ B and JNK pathways (17, 19).

In this study, we report that although XEDAR lacks a death domain, it nevertheless possesses the ability to induce programmed cell death. Unlike Fas and the TRAIL receptors, XEDAR does not directly bind to the death adaptors FADD, TRADD, or RIP but activates caspase 8 via the formation of a complex containing FADD, caspase 8, and caspase 10. Our results suggest that XEDAR may represent an early stage in the evolutionary history of death receptors and may play a role in the mediation of apoptosis during development and in adult life.

#### MATERIALS AND METHODS

**Cell Lines and Reagents**—293FLAG-XEDAR cells and expression plasmids encoding FLAG-XEDAR, its carboxyl-terminal deletion mutant N172, crmA, FLAG-DR4, FLAG-TNFR1, caspase 8 C360S, and cFLIP<sub>L</sub>/MRT1a have been described previously (19, 20). Rabbit polyclonal antibodies against RIP, TRAF1, TRAF3, TRAF6, and  $\beta$ -actin and goat polyclonal antibodies against FADD, RICK, caspase 8, and TRADD were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase 8, cleaved caspase 3, caspase 9, cleaved poly(ADP-ribose) polymerase, and BID were obtained from Cell Signaling (Beverly, MA). FLAG and control mouse IgG beads, mouse monoclonal anti-FLAG (M2)-horseradish peroxidase, and tubulin were obtained from Sigma. Caspase assay substrates DEVD-AFC, IETD-AFC, and LEHD-AFC and cell-permeable caspase inhibitors and were purchased from Enzyme Systems and Calbiochem (La Jolla, CA). Recombinant human EDA-A2 and TNF $\alpha$  were obtained from R&D Systems and also generated in SF9 cells as described earlier (19). XEDAR-Fc and EDAR-Fc were generated as described previously (19, 20). TNFR1-Fc and a neutralizing antibody against human TNF $\alpha$  were obtained from Peprotech Inc. (Rocky Hill, NJ).

**Co-immunoprecipitation Assays**—For studying *in vivo* interactions,  $5 \times 10^7$  293F-XEDAR-L cells were treated with control supernatant or EDA-A2 for 10 min. Cells were subsequently lysed in 5 ml of buffer A (20 mM sodium phosphate (pH 7.4), 150 mM NaCl) containing 5% glycerol, 1% Triton X-100, and 1 EDTA-free mini-protease inhibitor tablet/10 ml (Roche Applied Science). Cell lysates were precleared with mouse IgG beads and then incubated for 1 h at 4 °C with 50  $\mu$ l of FLAG beads precoated with a supersaturated casein solution. Beads were washed twice with buffer A, once with a high salt wash buffer (buffer A + 500 mM NaCl), and again with buffer A. Bound proteins were eluted by boiling, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blot. Co-immunoprecipitation of caspase 8 complex was performed using 2  $\mu$ g of an anticaspase 8 antibody (Santa Cruz Biotechnology) and essentially was as described previously (5).

**Caspase Activation Assay**—293F-XEDAR-L cells were left untreated (control) or treated with EDA-A2 or TRAIL (50 ng/ml) for different time intervals and subsequently lysed in buffer C (50 mM HEPES, pH 7.4, 1 mM EDTA, 10 mM, 10 mM dithiothreitol, 10% glycerol, and 0.1% CHAPS). Caspase activity was measured in a 100- $\mu$ l reaction volume containing cell lysate (20  $\mu$ g of total protein) and 5  $\mu$ M AFC-coupled peptide substrates in buffer D (buffer C containing 100 mM NaCl). The reaction was followed at 37 °C by SpectraFluor using 400 nm excitation and 510-nm emission filters. Caspase activities were estimated by measuring the turnover of substrates DEVD-AFC (caspase 3), IETD-AFC (caspase 8), and LEHD-AFC (caspase 9) (Enzyme Systems).

**Apoptosis Assays**—Cells ( $1.5\text{--}3 \times 10^5$ ) were transfected with the empty vector, XEDAR, TNFR1, or Fas (500 ng) along with  $\beta$ -lactosidase- and green fluorescent protein-encoding plasmids (75 ng each) using calcium phosphate (293T and 293F) or LipofectAMINE (L-929 cells). Cells were examined under a fluorescent microscope and photographed 36 h after transfection or were stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside as described previously (21).  $\beta$ -Galactosidase-positive cells were counted for viable and apoptotic cells based on their morphology. For all ligand-induced cell death experiments, stable cells expressing XEDAR were treated with EDA-A2 or TRAIL for 12 h and then stained with Hoechst 33342 and/or YOPRO-1. Cells were visualized by phase contrast and fluorescence microscopy. Approximately 300 cells were counted for each treatment ( $n = 3$ ) from three randomly selected fields, and the mean was used to calculate the percentage of apoptosis. Each experiment was repeated at least three times. Cell-permeable synthetic caspase inhibitors Boc-D-fmk, zVAD-fmk, zDEVD-fmk, zVDVAD-fmk, zVEID-fmk, zIETD-fmk, and zLEHD-

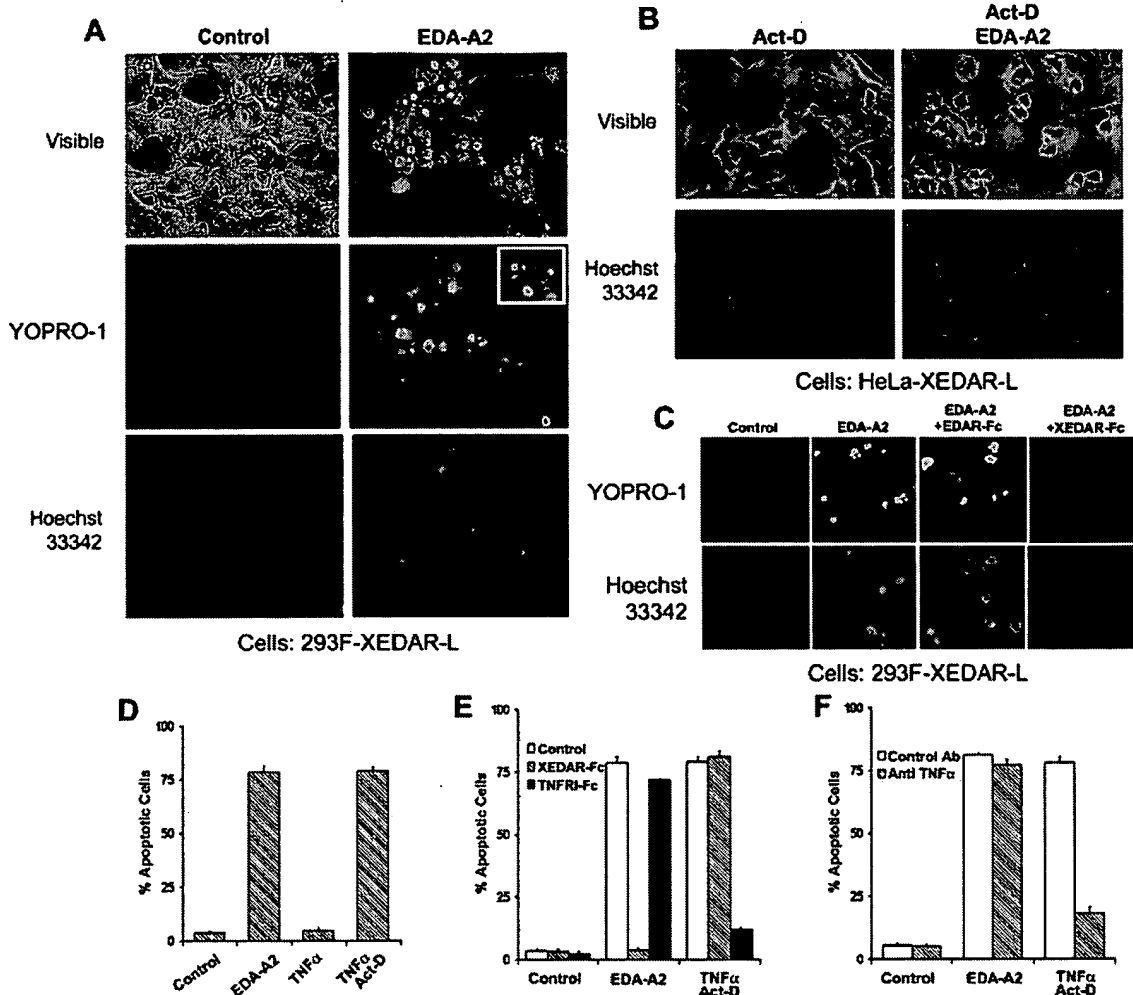
fmk were dissolved in ME<sub>2</sub>SO and added to the cells just before treatment with the ligand. ME<sub>2</sub>SO was added to the control wells to the same final concentration. For experiments testing the involvement of TNF $\alpha$ /TNFR1 in EDA-A2-induced apoptosis, TNF $\alpha$ , TNFR1-Fc, and a neutralizing monoclonal antibody against TNF $\alpha$  were used at concentrations of 10 ng/ml, 0.2  $\mu$ g/ml, and 0.5  $\mu$ g/ml, respectively.

**Preparation and Transfection of siRNA**—siRNA oligonucleotides with two thymidine residues (dTdT) at the 3'-end of the sequence were designed against caspase 8 (sense, 5'-GCAAGAACCAUCAAGGAUdTdT-3'), FADD-1 (sense, 5'-CGUCAUAUGUGAUAUGUGdTdT-3'), FADD-2 (sense, 5'-CCGAGCUCAAGUCCUAUGdTdT-3'), TRADD (sense, 5'-AACUCCACUUGGCCUAUCUdTdT-3'), mouse TRADD (control) (sense, 5'-UUAACAAGGCUCUGCAGACdTdT-3'), and lamin A/C (sense, 5'-CUGGACUCCAGAAGAACAAdTdT-3') along with their corresponding antisense oligonucleotides. 293FLAG-XEDAR cells were transfected with 80 nm double-stranded RNA using calcium phosphate in each well of a 6-well plate and replated into wells of a 24-well plate 24 h posttransfection. 48–60 h posttransfection cells were treated with EDA-A2 or TRAIL (50 ng/ml) for another 12 h. An untreated replicate from each set was analyzed by Western blotting to confirm the down-regulation of the protein targeted by siRNAs.

#### RESULTS

**Recombinant EDA-A2 Induces Apoptosis in 293F Cells Expressing XEDAR**—To facilitate the characterization of XEDAR signaling, we recently generated a subclone of 293F (human embryonic kidney), designated 293FLAG-XEDAR, with stable expression of the NH<sub>2</sub>-terminal FLAG epitope-tagged XEDAR-L isoform (19). These cells were generated using retrovirally mediated gene transfer and show modest expression of XEDAR as determined by cell surface staining with an antibody against the FLAG tag. While studying the ability of EDA-A2 to induce the NF- $\kappa$ B pathway in these cells, we were surprised to find that a large number of cells were undergoing cell death (Fig. 1A). EDA-A2-treated cells demonstrated typical features of apoptosis, such as cell rounding, detachment, and fragmentation into small apoptotic bodies (Fig. 1A, *top*). They also readily stained with YOPRO-1, a cell-impermeable nuclear dye that stains only those cells that have lost membrane integrity (Fig. 1A, *middle*) (22). Furthermore, nuclear staining with YOPRO-1 and Hoechst 33342 dyes showed that the nuclei of EDA-A2-treated cells were condensed and fragmented, another characteristic of apoptotic cells (Fig. 1A, *inset*). We confirmed the apoptosis-inducing ability of EDA-A2 using several independent preparations of this protein prepared in our laboratory as well as a commercially available preparation (R&D Systems) (not shown). The cytotoxicity of EDA-A2 was specific for XEDAR-expressing cells as it had no effect on parental 293 cells or those infected with a control retroviral vector (not shown). Furthermore, EDA-A2-induced cell death could be specifically blocked by soluble XEDAR (XEDAR-Fc), whereas soluble EDAR (EDAR-Fc) was without any inhibitory effect (Fig. 1C). EDA-A2 also induced apoptosis in a stable clone of HeLa cells expressing XEDAR, although these cells required sensitization with actinomycin-D (Fig. 1B).

It has been reported that the cytotoxic effects of TNFR2, CD40, and CD30 are mediated by the endogenous production of TNF $\alpha$ , which activates TNFR1 in an autotropic or paratropic fashion (23). Therefore, we sought to determine the role of TNF $\alpha$ /TNFR1 signaling in the induction of apoptosis by EDA-A2. Unlike EDA-A2, treatment of 293FLAG-XEDAR cells with TNF $\alpha$  (in the absence of actinomycin-D) failed to induce significant apoptosis, thereby arguing against the possibility that EDA-A2 induces apoptosis via the production of endogenous TNF $\alpha$  (Fig. 1D). More importantly, blockage of TNF $\alpha$ /TNFR1 signaling by the use of soluble TNFR1 receptor (TNFR1-Fc) or a neutralizing antibody against TNF $\alpha$  had no significant effect on apoptosis induced by EDA-A2, while successfully blocking apoptosis induced by combined treatment with TNF $\alpha$  and actinomycin-D (Fig. 1, E and F). Taken together, the above re-



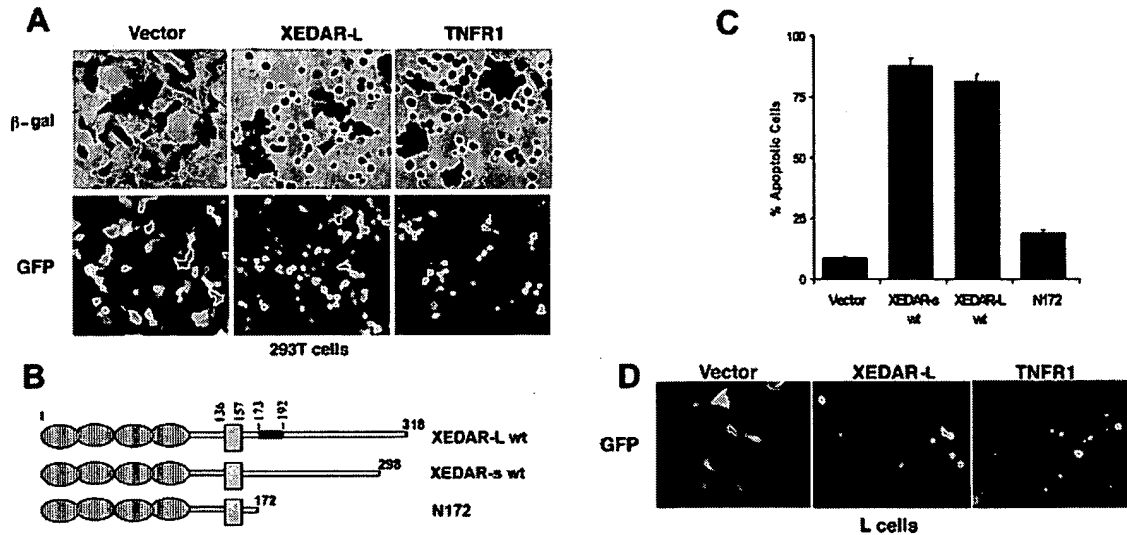
**FIG. 1. Induction of apoptosis by EDA-A2.** *A*, 293F-XEDAR-L cells were treated with control (phosphate-buffered saline) or EDA-A2 (50 ng/ml). 12 h after treatment, cells were stained with YOPRO-1, which stains the nuclei of dead cells that have lost their plasma membrane integrity, and Hoechst 33342 dyes, respectively. Cells were photographed under phase-contrast and fluorescent microscopes. The *inset* shows the nuclear morphology of EDA-A2-treated cells as demonstrated by YOPRO-1 staining. *B*, HeLa-XEDAR-L cells were treated with actinomycin-D (Act-D) (200 ng/ml) alone or along with EDA-A2 (50 ng/ml). 12 h after treatments, cells were stained with Hoechst 33342 and photographed under a fluorescent microscope. *C*, XEDAR-Fc completely blocks EDA-A2-induced cell death, whereas EDAR-Fc has no effect. 293F-XEDAR-L cells were treated with EDA-A2 alone or along with XEDAR-Fc or EDAR-Fc. 12 h after treatment, cells were stained with YOPRO-1 and Hoechst 33342 dyes, respectively, and photographed under a fluorescent microscope. *D*, treatment with TNF $\alpha$  alone fails to induce apoptosis in 293F-XEDAR-L cells. 293F-XEDAR-L cells were treated with control (phosphate-buffered saline), EDA-A2, TNF $\alpha$  (10 ng/ml), or TNF $\alpha$  plus actinomycin D (200 ng/ml). 12 h after treatment, cells were stained with Hoechst 33342, and the percentage of apoptotic cells was counted under a fluorescent microscope. *E* and *F*, blockage of TNF $\alpha$ /TNFR1 signaling fails to block EDA-A2-induced apoptosis. 293F-XEDAR-L cells were treated with control (phosphate-buffered saline), EDA-A2, or TNF $\alpha$  plus actinomycin D in the absence or presence of XEDAR-Fc or TNFR1-Fc (*E*) or a neutralizing antibody against TNF $\alpha$  (*F*), and the percentage of apoptotic cells was counted as described for *D*. Values shown are the mean  $\pm$  S.E. of a representative of two independent experiments performed in duplicate.

sults demonstrate that EDA-A2-induced apoptosis is not mediated via the production of TNF $\alpha$  and resultant signaling via TNFR1.

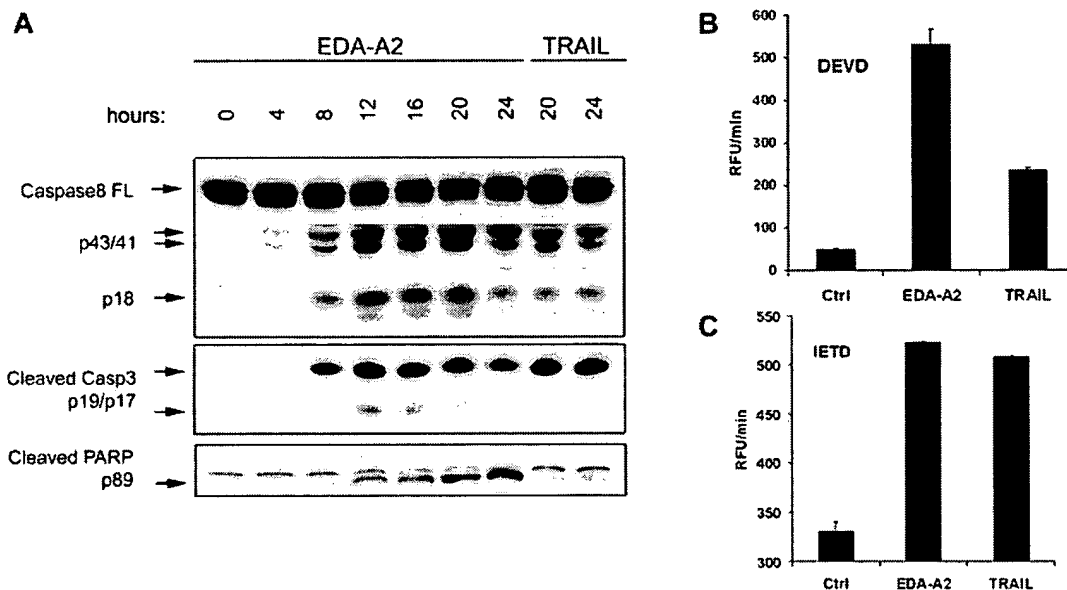
**Induction of Apoptosis by XEDAR**—Unlike the classical death receptors, XEDAR does not possess a death domain, and therefore, the ability of EDA-A2 to induce apoptosis in XEDAR-expressing cells was unexpected. Signaling via the receptors of the TNFR family can be activated in a ligand-independent fashion by overexpression-induced receptor aggregation (19). Therefore, we sought to determine whether overexpression of XEDAR by itself would induce apoptosis. As shown in Fig. 2, *A* and *C*, transient transfection of plasmids encoding full-length XEDAR-L or XEDAR-s isoforms (19), but not a carboxyl-terminal deletion mutant (N172), in 293T cells led to cellular rounding, condensation detachment, and fragmentation into apoptotic bodies, features suggestive of cell death. We obtained

essentially similar results upon transient transfection of XEDAR in mouse L-929 cells (Fig. 2*D*).

**Activation of Extrinsic Caspase Pathway during EDA-A2-induced Apoptosis**—The presence of nuclear fragmentation during EDA-A2-induced apoptosis pointed toward the involvement of caspases. Therefore, to test whether caspase activation is involved in XEDAR-induced apoptosis, we treated 293FLAG-XEDAR cells with EDA-A2 and TRAIL (positive control) and analyzed the cell lysates for cleavage of various caspases known to be involved in the induction of apoptosis. We readily detected cleavage of caspase 8 into p43, p41, and p18 fragments within 8 h of EDA-A2 treatment, although weak cleavage was evident within 4 h (Fig. 3*A*). Caspase 3 is one of the executioner caspases of the caspase cascade and is activated by caspase 8 during apoptosis induced by the death receptors. We detected significant cleavage of caspase 3 into its active fragments, p19



**FIG. 2. Transient transfection of XEDAR induces cell death.** *A*, 293T cells ( $1.5 \times 10^5$ ) were transfected with the indicated plasmids (500 ng) along with  $\beta$ -galactosidase ( $\beta$ -gal)- and green fluorescent protein (GFP)-encoding plasmids (75 ng of each). 36 h posttransfection, cells were examined under a fluorescent microscope and photographed or were fixed and stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside as described previously (21). XEDAR- and TNFR1-transfected cells have a dark rounded appearance and condensed nuclei and are becoming detached from the plate, whereas cells transfected with the empty vector have normal morphology. *B*, a schematic representation of XEDAR expression constructs. The XEDAR-L isoform differs from the XEDAR-s isoform by the presence of an additional 21 amino acids (amino acids 173–192) in the juxtamembrane region of the cytoplasmic domain. N172 is a carboxyl-terminal deletion mutant. *wt*, wild type. *C*, induction of apoptosis by XEDAR-L and XEDAR-s isoforms. 293T cells were transfected with the indicated plasmids along with pEGFP as described in *A*. Apoptotic cells were counted 36 h after transfection on the basis of their morphology. *D*, L-929 cells were transfected with the indicated plasmids along with an enhanced green fluorescent protein-encoding plasmid (75 ng) using LipofectAMINE (Invitrogen) and examined under a fluorescent microscope.

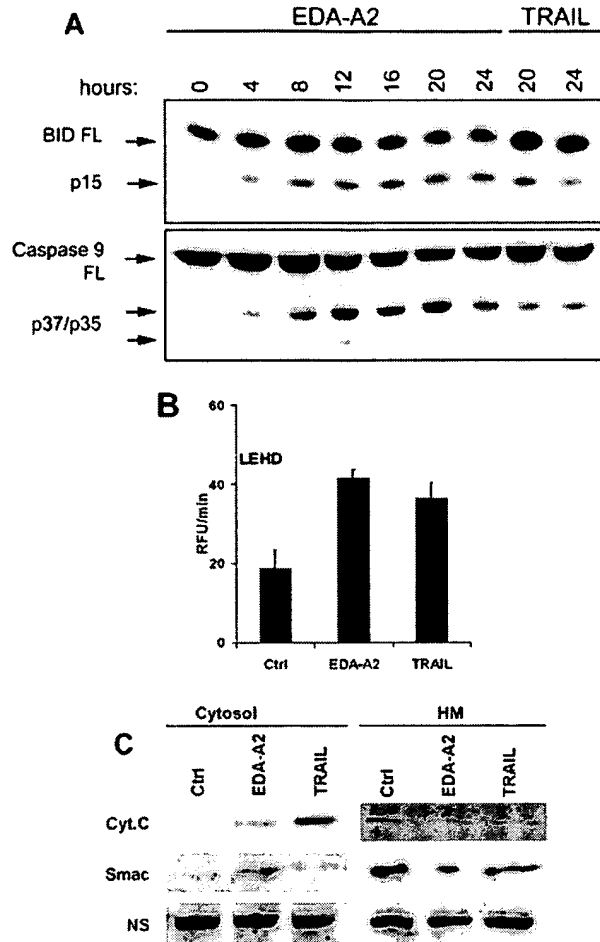


**FIG. 3. Activation of caspase 8 and caspase 3 during EDA-A2-induced apoptosis.** *A*, 293FLAG-XEDAR cells were left untreated (control) or treated with EDA-A2 (50 ng/ml) or TRAIL (50 ng/ml). After the indicated time intervals, cell lysates were prepared and analyzed for cleavage of caspase 8, caspase 3 (Casp3), and poly(ADP-ribose) polymerase (PARP) by Western blotting. *FL*, full-length. *B* and *C*, caspase 3 and caspase 8 activities were assayed in the cell extracts of 293FLAG-XEDAR cells treated with EDA-A2 or TRAIL for 20 h. Enzymatic activities were estimated as a function of time using a fluorometric assay to determine the turnover of peptide substrates DEVD-AFC (for caspase 3) and IETD-AFC (for caspase 8). The data shown are the mean relative fluorescent units (RFU)/min  $\pm$  S.E. of duplicate assays and are representative of three independent experiments. *Ctrl*, control.

and p17, following EDA-A2 treatment (Fig. 3A). EDA-A2 treatment also led to the cleavage of poly(ADP-ribose) polymerase, one of the caspase 3 substrates, indicating caspase 3 activation (Fig. 3A). We confirmed activation of caspase 8 and 3 in the lysates of EDA-A2-treated cells using their synthetic peptide substrates coupled with a fluorogen AFC (Fig. 3, *B* and *C*).

**Activation of Intrinsic (Mitochondrial) Caspase Pathway during EDA-A2-induced Apoptosis**—BID is a proapoptotic

member of the bcl2 family that is cleaved by caspase 8 during apoptosis induced by death domain-containing receptors of the TNFR family (24, 25). Truncated BID (tBID) then translocates to the mitochondria, where it induces release of cytochrome *c* into cytosol and subsequent activation of caspase 9. As shown in Fig. 4A, a cleaved fragment of BID (p15) appeared as early as 4 h after EDA-A2 treatment and correlated with the appearance of the cleaved fragments (p37/35) of



**FIG. 4. Activation of intrinsic caspase pathway during EDA-A2-induced apoptosis.** A, 293FLAG-XEDAR cells were left untreated (control) or treated with EDA-A2 (50 ng/ml) or TRAIL (50 ng/ml). After the indicated time intervals, cell lysates were prepared and analyzed for cleavage of caspase BID and caspase 9 by Western blotting. FL, full-length. B, caspase 9 activity was assayed in the cell extracts of 293FLAG-XEDAR cells treated with EDA-A2 or TRAIL for 20 h using LEHD-AFC as a substrate. Ctrl, control; RFU, relative fluorescent units. C, EDA-A2 induces release of cytochrome *c* (Cyt. C) and Smac from mitochondria. 293FLAG-XEDAR cells were treated with EDA-A2 or TRAIL for 12 h and then fractionated essentially as described previously (35) to separate the soluble cytosolic proteins from the heavy membrane (HM) fraction, which contained the mitochondria. Western blot analysis was then performed with antibody against cytochrome *c* (Pharmingen, BD Biosciences) and Smac (Oncogene). NS, nonspecific band.

caspase 9. Activation of caspase 9 following EDA-A2 treatment was further confirmed using a fluorogenic assay based on the cleavage of its synthetic peptide substrate (Fig. 4B). Treatment with EDA-A2 also resulted in the release of cytochrome *c* and Smac from the mitochondria into the cytosol (Fig. 4C). TRAIL was used as a positive control for the above experiments. Taken together, the above results demonstrate activation of both intrinsic and extrinsic cell pathways of caspase activation following EDA-A2 treatment.

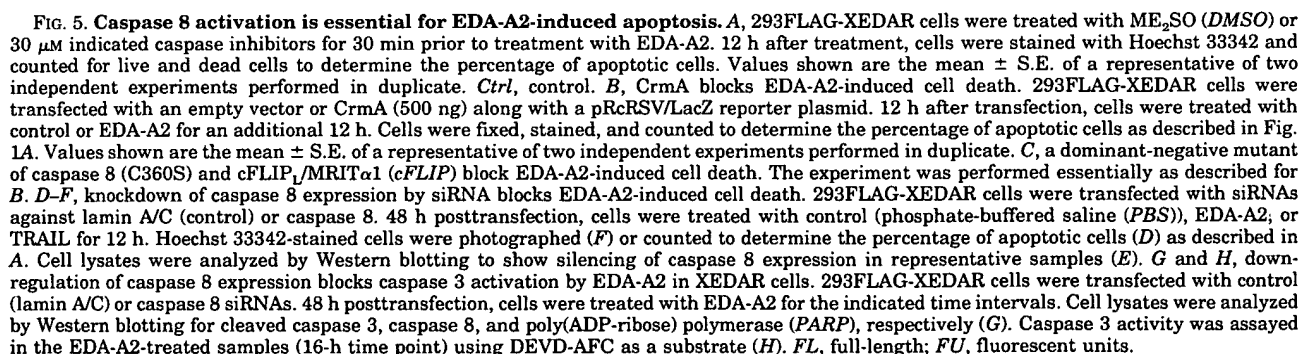
**Caspase Activation Is Required for EDA-A2-induced Apoptosis**—We next sought to determine whether caspases are functionally involved in EDA-A2-induced apoptosis. As shown in Fig. 5A, Boc-D-fmk and zVAD-fmk, two broad spectrum synthetic cell-permeable caspase inhibitors, effectively blocked EDA-A2-induced cell death. zIETD-fmk, a preferential inhibitor of caspase 8, was similarly very effective in blocking EDA-A2-induced apoptosis (Fig. 5A). In contrast, preferential inhib-

itors of caspase 3 (zDEVD-fmk) and caspase 9 (zLEHD-fmk) had only a partial inhibitory effect, whereas preferential inhibitors of caspase 2 (zVDVAD-fmk) and caspase 6 (zVEID-fmk) failed to significantly block EDA-A2-induced apoptosis (Fig. 5A). Finally, crmA, a cowpox virus-encoded preferential inhibitor of caspase 8 (26), also effectively blocked EDA-A2-induced apoptosis (Fig. 5B).

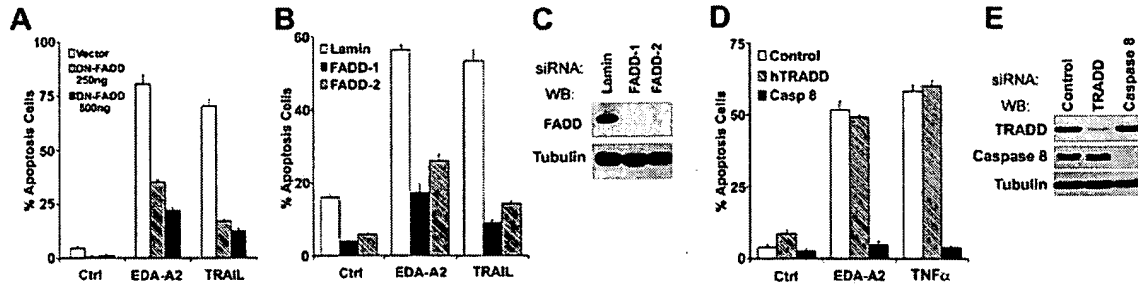
**Caspase 8 Is Essential for EDA-A2-induced Apoptosis**—We further analyzed the contribution of caspase 8 to XEDAR-induced apoptosis by using its dominant-negative mutant (caspase 8 C360S) and cFLIP<sub>L</sub>/MRIT $\alpha$ 1, a naturally occurring cytoplasmic inhibitor of caspase 8 (27, 28). As shown in Fig. 5C, both caspase 8 C360S mutant and cFLIP<sub>L</sub>/MRIT $\alpha$ 1 blocked XEDAR-induced apoptosis with efficiency comparable with that against Fas, a receptor known to use caspase 8 for induction of cell death. EDA-A2-induced apoptosis was also effectively blocked by siRNA-mediated down-regulation of caspase 8 expression. In fact, caspase 8 siRNA blocked cell death induced by EDA-A2 with efficiency comparable with that against TRAIL, a TNF family ligand known to require caspase 8 for the induction of apoptosis (Fig. 5, D–F). Interestingly, siRNA-mediated down-regulation of caspase 8 expression led to complete inhibition of caspase 3 activation, thereby demonstrating that activation of caspase 8 is required for activation of caspase 3 following EDA-A2 treatment (Fig. 5, G and H). Taken together, the above results establish caspase 8 as the apical caspase in EDA-A2/XEDAR-induced apoptosis and demonstrate that it plays a non-redundant and indispensable role in this process.

**Role of FADD in EDA-A2-induced Apoptosis**—FADD is a common mediator of cell death induced by the classical death domain-containing receptors of the TNFR family and is recruited to them either directly or via the intermediate adaptor protein TRADD (3, 8). We checked the involvement of FADD in XEDAR-induced cell death by using a dominant-negative mutant of FADD (DN-FADD) that lacks its death effector domain (29). We found that overexpression of DN-FADD blocked XEDAR-induced cell death in a dose-dependent fashion (Fig. 6A). However, dominant-negative FADD was slightly less effective in blocking cell death induced by EDA-A2 as compared with that induced by TRAIL (Fig. 6A). We used two different siRNAs directed against FADD to confirm its involvement in EDA-A2-induced cell death. As shown in Fig. 6, B and C, we observed significant inhibition of apoptosis upon EDA-A2 treatment in cells in which FADD expression had been silenced using either of the two siRNAs. In contrast a siRNA directed against lamin A/C failed to significantly block EDA-A2-induced apoptosis. However, consistent with our previous results using DN-FADD, we observed that siRNAs against FADD were slightly less effective in blocking EDA-A2-induced apoptosis as compared with TRAIL-induced apoptosis (Fig. 6B). Collectively, the above results suggest that although FADD is involved in EDA-A2-induced apoptosis, it might play a role distinct from its role in TRAIL-induced apoptosis.

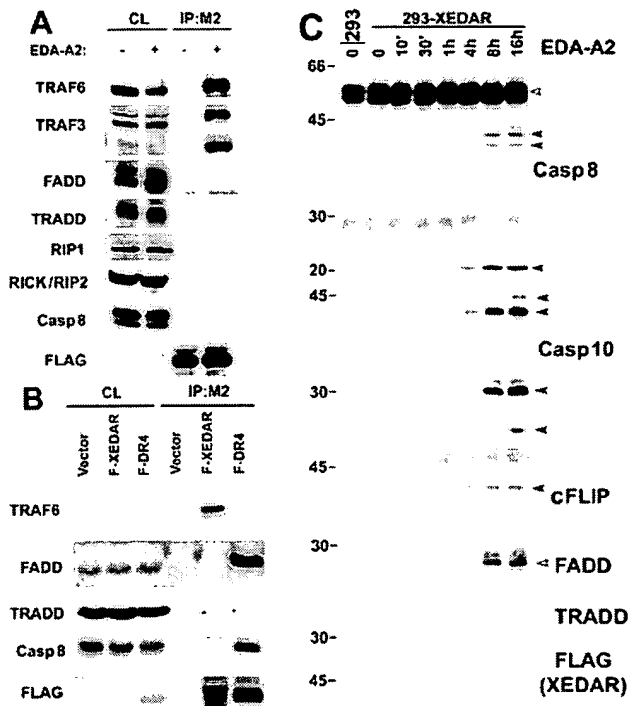
TRADD is a component of both complex I and II during TNFR1 signaling (5). Although TRADD is generally believed to be a key mediator of FADD recruitment during TNFR1 signaling, genetic evidence of its involvement in TNFR1-induced apoptosis is lacking so far. We tested the involvement of TRADD in EDA-A2- and TNF $\alpha$ -induced apoptosis using the siRNA approach. Remarkably, siRNA-mediated silencing of TRADD expression failed to significantly block EDA-A2- or TNF $\alpha$ -induced apoptosis in 293FLAG-XEDAR cells, while silencing of caspase 8 effectively did so (Fig. 6, D and E). These results argue against the involvement of TRADD in EDA-A2-induced apoptosis and suggest that its role in TNFR1-induced apoptosis may need re-examination.



tated samples was detected by Western blot analysis. Consistent with our previously published results (19), we readily detected an interaction between stably expressed FLAG-XEDAR and endogenous TRAF6 and TRAF3 upon treatment with EDA-A2 (Fig. 7A). However, under similar conditions, XEDAR failed to recruit endogenous FADD or TRADD (Fig. 7A). Similarly, we did not detect an interaction between XEDAR and protein kinases RIP1 or RICK/RIP2 (Fig. 7A). In an independ-



**FIG. 6. Role of FADD in EDA-A2-induced apoptosis.** A, DN-FADD protects cells from EDA-A2-induced cell death. 293FLAG-XEDAR cells ( $2 \times 10^5$ ) were transfected with an empty vector or different amounts (250 and 500 ng) of DN-FADD expression plasmid along with a  $\beta$ -galactosidase reporter plasmid in duplicate in each well of a 24-well plate. The total amount of transfected DNA was kept constant by adding empty vector. Cells were fixed and stained, and the percentage of apoptotic cells was determined based on criteria described in Fig. 2A. Values shown are the mean  $\pm$  S.E. of a representative of three independent experiments performed in duplicate. B, siRNA-mediated knockdown of FADD expression protects cells from EDA-A2-induced cell death. 293FLAG-XEDAR cells were transfected with the indicated siRNA duplexes. 40–60 h posttransfection, cells were treated with EDA-A2 or TRAIL. Apoptotic cells were determined based on Hoechst 33342 staining. Values shown are the mean  $\pm$  S.E. of a representative of three independent experiments performed in duplicate. C, Western blot (WB) analysis of cell lysates shows down-regulation of endogenous FADD expression by respective siRNAs. Lamin siRNA was used as a control. D, siRNA-mediated knockdown of TRADD expression fails to protect cells from EDA-A2- or TNF $\alpha$  (plus actinomycin-D)-induced cell death. The experiment was performed essentially as described for B except that actinomycin-D (200 ng/ml) was added to cells treated with TNF $\alpha$ . siRNA against caspase 8 was used as a positive control (Ctrl). E, Western blot analysis of cell lysates shows down-regulation of endogenous TRADD and caspase 8 expression by respective siRNAs.



**FIG. 7. Lack of recruitment of endogenous TRADD, FADD, or caspase 8 to XEDAR during EDA-A2 signaling.** A, 293FLAG-XEDAR cells were treated with control (–) and EDA-A2 (+) for 10 min. Total cell lysates (CL) were immunoprecipitated (IP) with FLAG beads (M2) (Sigma). Endogenously expressed co-immunoprecipitated proteins were detected by Western blot with the indicated antibodies. TRAF3 and -6 are recruited to XEDAR in a ligand-dependent manner, but FADD, TRADD, caspase 8, RIP1, or RICK/RIP2 fails to do so. B, 293T cells were transfected with an empty vector, FLAG-tagged XEDAR (F-XEDAR), or DR4 (F-DR4) plasmids. 24 h after transfection, cells were lysed, and total cell lysates were immunoprecipitated with FLAG beads. Endogenous proteins that co-immunoprecipitated with FLAG-XEDAR and FLAG-DR4 were detected by Western blotting with the indicated antibodies. C, formation of a secondary complex containing caspase 8 during EDA-A2-induced apoptosis. 293FLAG-XEDAR cells were treated with EDA-A2 for the indicated time intervals, and immunoprecipitation was performed using an anticaspase 8 antibody. Endogenously expressed co-immunoprecipitated proteins were detected by Western blot with the indicated antibodies. Cleaved proteins are indicated with filled arrowheads, whereas open arrowheads point to their full-length forms.

ent experiment, we transiently overexpressed FLAG-XEDAR in 293T cells and tested its interaction with endogenously expressed adaptor proteins and caspase 8. Again, we readily observed an interaction between overexpressed XEDAR and endogenously expressed TRAF6 but failed to see an interaction between overexpressed XEDAR and FADD, TRADD, or caspase 8 (Fig. 7B). We used overexpression of DR4 as a positive control in this experiment and, as expected, readily detected its interaction with endogenously expressed FADD and caspase 8 (Fig. 7B). Collectively the above results demonstrate that FADD, TRADD, RIP1, and RIP2 are not directly recruited to XEDAR during induction of cell death.

**Evidence for a Caspase 8, Caspase 10, and FADD-containing Secondary Complex during EDA-A2-induced Apoptosis—**Lack of recruitment of FADD and caspase 8 to XEDAR was reminiscent of signaling via TNFR1, in which caspase 8 and FADD form a secondary proapoptotic cytosolic complex (complex II) distinct from the receptor-containing membrane-associated complex (complex I) (5, 7). As such, we sought to determine whether signaling via EDA-A2 will similarly lead to the formation of a secondary complex between caspase 8 and FADD. For this purpose, we immunoprecipitated caspase 8 from the lysates of 293FLAG-XEDAR cells with and without treatment with EDA-A2 for different time intervals. No association between caspase 8 and FADD was found in the untreated cells (Fig. 7C). Association started 4 h poststimulation and increased further at the 8- and 16-h time points (Fig. 7C). Interestingly, recruitment of FADD to the caspase 8 complex corresponded to the time of its cleavage (Fig. 7C). We also detected cleavage fragments of caspase 10 in the caspase 8 complex at the 8- and 16-h time points (Fig. 7C). Caspase 8 inhibitor cFLIP<sub>L</sub> is processed in cells undergoing TNFR1-induced apoptosis (5). Interestingly, although no association of cFLIP<sub>L</sub> with caspase 8 was detected in untreated 293FLAG-XEDAR cells, a 43/41-kDa cleavage fragment of cFLIP<sub>L</sub> was found associated with caspase 8 in cells treated with EDA-A2 for 8 and 16 h, respectively (Fig. 7C). However, we failed to detect any full-length cFLIP<sub>L</sub> in the caspase 8 complex, suggesting that like the situation with Fas- and TNFR1-induced apoptosis, XEDAR-induced apoptosis is associated with complete processing of cFLIP<sub>L</sub>. Remarkably, no recruitment of XEDAR or TRADD was detected to the caspase 8-associated secondary complex at any of the time points (Fig. 7C). Taken together, the above results suggest that XEDAR-induced apo-



ptosis is mediated via the formation of a secondary complex between caspase 8, caspase 10, and FADD.

#### DISCUSSION

In this report we demonstrate that XEDAR, which lacks a death domain, is capable of inducing apoptosis. In this context, XEDAR resembles the recently isolated *Drosophila* TNFR homolog Wengen, which also lacks a death domain but is capable of inducing apoptosis via a caspase-dependent mechanism (30, 31). Wengen-induced apoptosis is also dependent on the JNK pathway and can be inhibited by the JNK phosphatase Puckered (31). We have demonstrated previously that EDA-A2/XEDAR signaling can induce JNK activation (19), and it is conceivable that akin to Wengen, JNK activation also contributes to XEDAR-induced apoptosis. XEDAR may represent an early stage in the evolutionary history of the death receptors prior to the emergence of the death domain, which probably appeared subsequently to hasten and augment the apoptotic process. Consistent with the above hypothesis, we have observed that EDA-A2 is a relatively weak and slow inducer of apoptosis as compared with TRAIL. Similarly, we and others have reported previously that Wengen and Eiger are relatively weak inducers of apoptosis in the *Drosophila* cells (31–33).

The evolutionary relationship between XEDAR and Wengen is also supported by structural similarities between the two receptors and their respective ligands. In addition to the lack of a death domain in their cytoplasmic domains, both XEDAR and Wengen share structural similarities in their NH<sub>2</sub>-terminal regions. Thus, XEDAR is a type III transmembrane protein and lacks an NH<sub>2</sub>-terminal signal peptide, differing in this aspect from the majority of mammalian TNF family receptors, which are type I transmembrane proteins. Similarly, Wengen is believed to either lack an NH<sub>2</sub>-terminal signal peptide or to possess an atypical signal peptide (30, 31). The structural similarity between XEDAR and Wengen also extends to their ligands, EDA-A2 and Eiger, respectively. Both Eiger and EDA are type II transmembrane proteins that differ from other mammalian TNF family ligands in possessing a relatively long extracellular domain that contains a unique juxtamembrane subdomain not seen in other ligands of this family. This domain is rich in aspartic acid residues in the case of Eiger and glycine residues in the case of EDA and has been postulated to help in ligand oligomerization (31–33).

A recent study reported that XEDAR-deficient mice were indistinguishable from their wild type littermates (18). Although this study did not focus on the induction of apoptosis in XEDAR-null animals, its negative results may be explained by the fact that XEDAR plays a redundant role in the regulation of apoptosis during embryonic development. Consistent with the above hypothesis, we and others have reported previously that overexpression of EDAR and TAJ/Troy, two homologs of XEDAR that are also highly expressed during embryogenesis, can induce cell death (20, 21, 34). Finally, it is possible that XEDAR induces apoptosis only in limited organs/tissues and in specific cellular contexts and that subtle defects in apoptosis induction in XEDAR-deficient animals might fail to yield an overt developmental phenotype.

Transgenic expression of soluble EDA-A2, but not EDA-A1, was reported recently to lead to the birth of thin and listless animals that died within 1 month of birth (18). Histological analysis of affected animals revealed multifocal skeletal muscle degeneration, which was absent in EDA-A2-transgenic mice lacking XEDAR expression, thereby suggesting that it was a direct consequence of XEDAR signaling and not a nonspecific effect of excessive EDA-A2 protein (18). Although the downstream events involved in EDA-A2-induced myodegeneration

are unclear at the present, it is conceivable that caspase activation and induction of cell death play a role in this process. EDA-A2-induced caspase activation and cell death may also play a role in tissue remodeling/differentiation in organs showing XEDAR expression in adult life. Finally, EDA-A2-induced apoptosis may be exploited for the treatment of cancers showing XEDAR expression.

In this report, we present evidence that XEDAR utilizes a novel mechanism for activation of the caspase cascade and induction of apoptosis. Like the situation with TNFR1, activation of caspase 8 during XEDAR signaling is achieved via the formation of a secondary complex containing caspase 8, caspase 10, and FADD, which does not contain the receptor. However, unlike the situation with TNFR1, the proapoptotic complex formed during XEDAR-induced cell death lacks TRADD. Thus, our study suggests that TRADD is not essential for the formation of complex II containing caspase 8, caspase 10, and FADD. In this context, it is important to point out that although TRADD has been postulated to play a role in TNFR1-induced apoptosis, genetic evidence to support its involvement in this process has been lacking so far. In the light of our results suggesting that XEDAR signaling can lead to the formation of complex II without TRADD and that siRNA-mediated silencing of TRADD expression failed to block TNF $\alpha$ -induced apoptosis, the role of TRADD in TNFR1-induced apoptosis may require re-examination.

We have observed a difference in the relative abilities of DN-FADD and FADD siRNAs to block EDA-A2- versus TRAIL-induced apoptosis. This discrepancy might be explained by the fact that although FADD is involved in caspase 8 activation during both EDA-A2- and TRAIL-induced apoptosis, it does so via two distinct complexes. Presumably, a greater amount of FADD is required for the formation of the death-inducing signal complex and subsequent caspase 8 activation during TRAIL signaling as compared with the formation of complex II during EDA-A2-induced apoptosis. It is also conceivable that although FADD may be absolutely required for DISC formation and subsequent caspase 8 activation via FasL and TRAIL, it may facilitate the formation and/or activity of the caspase 8-containing secondary complex following EDA-A2 signaling without being absolutely essential for this process.

Although our study does not reveal the nature of the trigger that could potentially lead to the formation of the secondary complex capable of activating caspase 8 during XEDAR signaling, recent studies of TNFR1-induced apoptosis may provide some clues. It has been proposed that the relative level of caspase regulator cFLIP<sub>L</sub> may control the activation of caspase 8 in complex II during TNFR1-induced apoptosis by controlling the access of caspase 10 to caspase 8 (5, 9). In the current study, we demonstrate that caspase 8-associated cFLIP<sub>L</sub> is completely cleaved to its 43/41-kDa fragment during EDA-A2-induced apoptosis, and its forced overexpression blocks EDA-A2-induced cell death. Thus, cFLIP<sub>L</sub> may be the final arbiter of caspase 8 activation during XEDAR signaling. Finally, in the case of TNFR1 signaling, it has been proposed that the dissociation of complex II from the receptor and its localization in the cytosol or its association with the cytoskeleton may facilitate caspase recruitment and activation by potentially bringing the complex in proximity to apoptotic proteins (9). It is conceivable that a similar mechanism may be operative during XEDAR-induced apoptosis.

#### REFERENCES

- Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) *Cell* 104, 487–501
- Baud, V., and Karin, M. (2001) *Trends Cell Biol.* 11, 372–377
- Ashkenazi, A., and Dixit, V. M. (1998) *Science* 281, 1305–1308
- Chen, G., and Goeddel, D. V. (2002) *Science* 296, 1634–1635
- Micheau, O., and Tschopp, J. (2003) *Cell* 114, 181–190
- Legler, D. F., Micheau, O., Doucey, M. A., Tschopp, J., and Bron, C. (2003)



- Immunity* 18, 655–664
7. Harper, N., Hughes, M., MacFarlane, M., and Cohen, G. M. (2003) *J. Biol. Chem.* 278, 25534–25541
  8. Peter, M. E., and Krammer, P. H. (2003) *Cell Death Differ.* 10, 26–35
  9. Barnhart, B. C., and Peter, M. E. (2003) *Cell* 114, 148–150
  10. Peter, M. E. (2000) *Cell Death Differ.* 7, 759–760
  11. Mikkola, M. L., and Thesleff, I. (2003) *Cytokine Growth Factor Rev.* 14, 211–224
  12. Pinheiro, M., and Freire-Maia, N. (1994) *Am. J. Med. Genet.* 53, 153–162
  13. Kere, J., Srivastava, A. K., Montonen, O., Zonana, J., Thomas, N., Ferguson, B., Munoz, F., Morgan, D., Clarke, A., Baybayan, P., Chen, E. Y., Ezer, S., Saarialho-Kere, U., de la Chapelle, A., and Schlessinger, D. (1996) *Nat. Genet.* 13, 409–416
  14. Srivastava, A. K., Pispas, J., Hartung, A. J., Du, Y., Ezer, S., Jenks, T., Shimada, T., Pekkanen, M., Mikkola, M. L., Ko, M. S., Thesleff, I., Kere, J., and Schlessinger, D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 13069–13074
  15. Mikkola, M. L., Pispas, J., Pekkanen, M., Paulin, L., Nieminen, P., Kere, J., and Thesleff, I. (1999) *Mech. Dev.* 88, 133–146
  16. Bayes, M., Hartung, A. J., Ezer, S., Pispas, J., Thesleff, I., Srivastava, A. K., and Kere, J. (1998) *Hum. Mol. Genet.* 7, 1661–1669
  17. Yan, M., Wang, L. C., Hymowitz, S. G., Schilbach, S., Lee, J., Goddard, A., de Vos, A. M., Gao, W. Q., and Dixit, V. M. (2000) *Science* 290, 523–527
  18. Newton, K., French, D. M., Yan, M., Frantz, G. D., and Dixit, V. M. (2004) *Mol. Cell. Biol.* 24, 1608–1613
  19. Sinha, S. K., Zachariah, S., Quinones, H. I., Shindo, M., and Chaudhary, P. M. (2002) *J. Biol. Chem.* 277, 44953–44961
  20. Kumar, A., Eby, M. T., Sinha, S., Jasmin, A., and Chaudhary, P. M. (2001) *J. Biol. Chem.* 276, 2668–2677
  21. Eby, M. T., Jasmin, A., Kumar, A., Sharma, K., and Chaudhary, P. M. (2000) *J. Biol. Chem.* 275, 15336–15342
  22. Idziorek, T., Estaquier, J., De Bels, F., and Ameisen, J. C. (1995) *J. Immunol. Methods* 185, 249–258
  23. Grell, M., Zimmermann, G., Gottfried, E., Chen, C. M., Grunwald, U., Huang, D. C., Wu Lee, Y. H., Durkop, H., Engelmann, H., Scheurich, P., Wajant, H., and Strasser, A. (1999) *EMBO J.* 18, 3034–3043
  24. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) *Cell* 94, 491–501
  25. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) *Cell* 94, 481–490
  26. Ekert, P. G., Silke, J., and Vaux, D. L. (1999) *Cell Death Differ.* 6, 1081–1086
  27. Han, D. K., Chaudhary, P. M., Wright, M. E., Friedman, C., Trask, B. J., Riedel, R. T., Baskin, D. G., Schwartz, S. M., and Hood, L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 11333–11338
  28. Irmeler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) *Nature* 388, 190–195
  29. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) *Cell* 81, 505–512
  30. Kanda, H., Igaki, T., Kanuka, H., Yagi, T., and Miura, M. (2002) *J. Biol. Chem.* 277, 28372–28375
  31. Kaupilla, S., Maaty, W. S., Chen, P., Tomar, R. S., Eby, M. T., Chapo, J., Chew, S., Rathore, N., Zachariah, S., Sinha, S. K., Abrams, J. M., and Chaudhary, P. M. (2003) *Oncogene* 22, 4860–4867
  32. Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Aigaki, T., and Miura, M. (2002) *EMBO J.* 21, 3009–3018
  33. Moreno, E., Yan, M., and Basler, K. (2002) *Curr. Biol.* 12, 1263–1268
  34. Wang, Y., Li, X., Wang, L., Ding, P., Zhang, Y., Han, W., and Ma, D. (2004) *J. Cell Sci.* 117, 1525–1532
  35. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) *Mol. Cell* 8, 613–621

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**